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Remarks

Applicants thank the Examiner for the telephone interview conducted on June 21, 2004.

Applicants acknowledge the Examiner's indication that claims 23-24, 27-28, 32, 36, 46-48 and 58-60 would be allowed if rewritten so as not to depend from rejected claims, and that claims 13-15 and 21-60 are deemed free of the prior art.

Claims 13-15 and 21-60 are currently pending in the application. Claims 13 and 15 have been canceled, and claims 14, 21, 23-25, 27, 32, 36 and 58 have been amended. The amendments to claims 32, 36 and 58 merely change the dependency of the claims. The amendments to claims 23, 24 and 27 merely change the language used in the claims. Support for the claim amendments to claims 14, 21, 23, 24 and 27 may be found throughout the specification, including the claims as originally filed. In particular, support for the amendment to claim 14 ("encodes a protein having sulfotransferase activity") may be found, e.g., at pages 53, line 17. Support for the amendments to claims 21 and 25 ("95%") may be found, e.g., at page 54, line 21. No new matter has been added.

Amendment or cancellation of claims should in no way be construed as an acquiescence to any of the Examiner's rejections. The amendments to and cancellations of the claims are being made solely to expedite prosecution of the present application. Applicants reserve the right to further prosecute claims drawn to all subject matter disclosed in the instant patent application or in a continuation thereof.

Rejection of claims* 13-15, 21-22, 25-26, 29-31, 33-35, 37-45 and 49-57 *under 35 U.S.C. §112, first paragraph

Written Description

Claims 13-15, 21-22, 25-26, 29-31, 33-35, 37-45 and 49-57 were rejected for allegedly "contain[ing] subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor(s), at the time the application was filed, had possession of the claimed invention." Applicants respectfully traverse this rejection.

Claims 13 and 15 have been canceled, thereby rendering their rejection moot.

Regarding claims 21-22 and 25-26, the Examiner indicates that "at least '90%' or '99%' identity do not find support in the specification as filed and thus constitutes new matter." Applicants respectfully submit that claims 21 and 25 have been amended to recite "95%" instead of "90%." Furthermore, support for the terms "90%", "95%" and "99%" can be found, e.g., at page 54, line 21, of the specification. Thus, these terms do not constitute new matter.

Regarding claims 13-15, 21-22, 25-26, 29-31, 33-35, 37-45 and 49-57, the Examiner indicates that they fail to comply with the written description requirement allegedly because

> the specification ... does not describe the structural features of SEQ ID NO: 15 that are retained by sequences that hybridize to SEQ ID NO: 15 and that encode a polypeptide having sulfotransferase activity. The specification additionally does not describe the structural features of SEQ ID NO: 15 that are retained by sequences encoding polypeptides having at least 90% or 99% identity with SEQ ID NO: 16 and having sulfotransferase activity. The specification further does not describe the structural features of SEQ ID NO: 15 that are retained by sequences having at least 90% or 99% identity with SEQ ID NO: 15 and that encode polypeptides having sulfotransferase activity. The structural features unique to the claimed genus of sequences are not described, and the single nucleotide sequence disclosed in the specification (SEQ ID NO: 15 encoding a single amino acid sequence (SEQ ID NO: 16) having a moderate level of homology to known flavonol sulfotransferases does not constitute a representative number of species adequate to support the description of the multiple sequence variants of SEQ ID NOs: 15 and 16 encompassed by the claimed genus of sequences.

Applicants respectfully traverse these statements. Regarding claim 14, which is drawn to an isolated nucleic acid which hybridizes under stringent conditions including a wash step in 0.2 x SSC at 65 °C to a nucleic acid consisting of the nucleotide sequence set forth in SEQ ID NO: 15, wherein the isolated nucleic acid encodes a protein having sulfotransferase activity, Applicants assert that such isolated nucleic acids retain the structural features of SEQ ID NO: 15. The Synopsis of the Written Description Guidelines, which are available on the U.S. Patent and Trademark Office's website, specifically provide an example (Example 9) of a similar claim and indicates that the claim meets the written description requirement even though only a single species is disclosed. In particular, Example 9 states that a claim that is drawn to "an isolated nucleic acid that specifically hybridizes under highly stringent conditions to the complement of the sequence set forth in SEQ ID NO: 1, wherein said nucleic acid encodes a protein that binds to

a dopamine receptor and stimulates adenylate cyclase activity" meets the written description requirement. The Synopsis explains that "a person of skill in the art would not expect substantial variation amount species encompassed within the scope of the claims because the highly stringent hybridization conditions set fort in the claim yield structurally similar DNAs" and that "[t]hus, a representative number of species is disclosed, since highly stringent hybridization conditions in combination with the coding function of DNA and the level of skill and knowledge in the art are adequate to determine that applicant was in possession of the invention." Thus, in view of the similarity of the claim in the Synopsis and claim 14, Applicants respectfully submit that claim 14 also meets the written description requirements.

Regarding claims reciting a 95 or 99% identity with SEQ ID NO: 15 or 16, Applicants respectfully submit the following. As indicated at page 29 of the specification, phenol sulfotransferases have at least about 60% identity in amino acid sequences. As further stated at page 29, certain amino acid sequences of these enzymes are particularly well conserved throughout phylogeny. These conserved regions can be grouped into 4 blocks of conserved amino acid sequences, referred to as conserved regions I-IV. These regions are shown in Figure 5, which represents a sequence alignment of the amino acid sequences of 5 sulfotransferases, including the *Z. marina* sulfotransferase. As further described in the specification, regions I and IV appear to encode the binding domain to 3'-phosphoadenosine-5'-phosphosulfate (PAPS), the substrate for the sulfonation reaction. These two regions comprise the consensus sequence TYPKSGT(N/T)W-X190-210-RKGXXGDWKXXFT. Page 29 further states that this sequence motif occurs in virtually all known sulfotransferase proteins. As can be seen in Figure 5, the *Z. marina* sulfotransferase also shares homology with the other sulfotransferases in these four regions.

Applicants further submit herewith two articles, Marsolais and Varin (1998) *Chemico-Biological Interactions* 109:117 and Marsolais and Varin (1995) *J. Biol. Chem.* 270:30458, which further describe structure-function relationships of flavonol sulfotransferases. These two articles discuss the role of particular amino acids in the conserved domains. For example, the 1998 paper indicates that Lys59 in region I is involved in affinity for PAPS and catalysis (page 119). The paper also indicates that Arg140 in region II is involved in cosubstrate binding and its mutation results in loss of catalytic activity and binding to PAPS (page 120). The paper further indicates that the strongly conserved Arg276 in domain IV is involved in the formation of the

enzyme-PAPS complex and participates in product binding, suggesting that it may be involved in catalysis (page 120). The *Z. marina* sulfotransferase also has these three conserved amino acids, corresponding to residues 83, 172 and 302 in SEQ ID NO: 16, respectively (see Figure 5).

Thus, Applicants respectfully submit that the structural features of SEQ ID NO: 15 that are retained by sequences having 95 and 99% identity with SEQ ID NO: 15 or 16 and that encode polypeptides having sulfotransferase activity are described in the specification and in the art that was available at the time the application was filed.

Thus, reconsideration and withdrawal of the rejection of claims 13-15, 21-22, 25-26, 29-31, 33-35, 37-45 and 49-57 for lack of written description is respectfully requested.

Enablement

While the Examiner acknowledges that the specification is "enabling for an isolated nucleic acid of SEQ ID NO:15 and an isolated nucleic acid encoding SEQ ID NO:16, as well as vectors, cells and plants comprising said isolated nucleic acids," claims 13-15, 21-22, 25-26, 29-31, 33-35, 37-45 and 49-57 were rejected allegedly because "the specification does not reasonably provide enablement for other nucleotide or amino acid sequences, or for vectors, cells and plants comprising other nucleotide or amino acid sequences." Applicants respectfully traverse this rejection.

As described above, there was, at the time of filing considerable information regarding the structure-function relationship of sulfotransferases, and the *Z. marina* sulfotransferase exhibits significant homologies in the regions known to be important for the activity of sulfotransferases. Accordingly, at the time of filing of the application, a person of skill in the art would have known where modifications to SEQ ID NO: 15 could be made without affecting the sulfotransferase activity of the protein.

Thus, reconsideration and withdrawal of the rejection of claims 13-15, 21-22, 25-26, 29-31, 33-35, 37-45 and 49-57 for lack of enablement is respectfully requested.

Conclusion

Applicants believe that the claims now pending are in condition for allowance. If a telephonic conference with the Applicant would be helpful in expediting prosecution of the instant application, the Examiner is invited to call Applicants' Attorney at the telephone number provided below.

The Commissioner is hereby authorized to credit any overpayment or charge any deficiencies to Deposit Account Number **06-1448, Reference CEA-007.01**

Respectfully submitted,

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August 2, 2004

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Recent developments in the study of the structure-function relationship of flavonol sulfotransferases

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Abstract

With the rapid proliferation of sulfotransferase (ST) cDNA sequences in the last 5 years, consensus sequences were identified in four conserved regions. The association of these regions with substrate binding or catalysis was tested in several site-directed mutagenesis studies. Due to their strict substrate and position specificities, the flavonol 3- and 4′-STs represent an advantageous model system for the study of the structure-function relationship of cytosolic STs. Using a combination of chimeric and site-directed mutant proteins, a domain was identified containing all the determinants responsible for the substrate specificity of these enzymes, and characterized amino acid residues conserved in all cloned STs that are involved in substrate binding and catalysis. This paper summarizes the results of these studies. © 1998 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Sulfotransferase; Chimeric; Site-directed mutant proteins

1. Introduction

Cytosolic sulfotransferases (ST) catalyze the transfer of a sulfuryl group from an activated nucleotide donor, 3′-phosphoadenosine 5′-phosphosulfate (PAPS), to appropriate hydroxyl or amino groups of acceptor substrates. An ordered bi-bi

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kinetic mechanism where PAPS is the first substrate to bind and 3′-phosphoadenosine 5′-phosphate (PAP) is the last product to be released from the enzyme has been determined for most cytosolic STs, suggesting a direct sulfate transfer without the involvement of a sulfo-enzyme intermediate. Nucleophilic attack at the sulfuryl group is the most likely mechanism of transfer, but it is not known whether this occurs via an associative or dissociative process.

Progress in understanding the structure-function relationship of STs has been limited by the fact that their 3-D structure has not been resolved. However, in the last few years a large number of cDNA clones coding for STs of different specificities have been isolated from various organisms, allowing to initiate studies on the structure-function relationship of these enzymes. The comparison of the deduced amino acid sequence of ST enzymes of animal, plant and bacterial origin has revealed significant homology, and four conserved regions have been identified (Fig. 1). Since these regions could participate in shared functions of STs, they represent rational targets to initiate studies on the structure-function relationship of these enzymes. Various approaches involving affinity labeling, site-directed mutagenesis and the construction of chimeric enzymes have been used, resulting in the identification of protein regions and amino acid residues involved in substrate binding and catalysis. The plant flavonol STs have been chosen as a model system to study the structure-function relationship of STs because of their strict substrate and position specificities, and this paper describes the results of these studies.

2. PAPS binding site and catalysis

Targets for site-directed mutagenesis of the flavonol 3-ST were chosen based on the assumption that basic amino acid side chains could interact with the negatively charged phosphate and sulfate groups of the cosubstrate. This is known to be the case for other nucleotide utilizing enzymes, such as kinases, which catalyze a reaction highly analogous to sulfation. In addition, STs do not require divalent cations for their catalytic activity. In kinases, a Mg^{2+} ion interacts with the

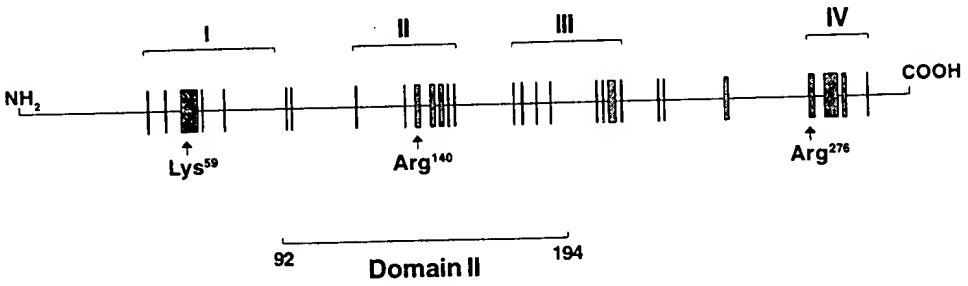

Fig. 1. Schematic representation of the conserved regions among STs. The backbone corresponds to a protein with 312 AA (flavonol 3-ST). The boxes represent conserved residues. Roman numbers refer to the conserved regions previously described. Numbers refer to the sequence of the flavonol 3-ST.

phosphate groups of the nucleotide. It is reasonable to assume that in STs, positively charged amino acid side chains could play a similar function as the Mg^{2+} ion in kinases. The involvement of basic amino acids is supported by previous chemical modification and affinity labeling studies of phenol STs which implicated arginine, lysine and possibly histidine residues as participating in cosubstrate binding and catalysis [1]. In a systematic approach, amino acid residues were selected within the conserved regions I, II and IV as targets for the site-directed mutagenesis of the flavonol 3-ST.

The conserved region I is located near the N-terminal end of STs (Fig. 1). A recent amino acid sequence comparison of cloned STs allows to define the consensus sequence of region I as (P,A)K(S,T)G(T,S)(T,N)W, where residues in parentheses represent alternatives for a single position. Recently, affinity labeling experiments of the rat aryl ST IV with the nucleotide analog ATP dialdehyde allowed the identification of two labeled amino acid residues, Lys^{65} and Cys^{66} [2]. However, these amino acids are only conserved in the phenol and estrogen ST families, and site-directed mutagenesis of the corresponding cysteine to serine in the human phenol-preferring phenol ST indicated that it is not involved in substrate binding or catalysis [3]. In view of the proximity of the labeled amino acids to region I, it was proposed that the latter may be involved in the interaction with the cosubstrate [2].

The characterization of Lys^{59} in region I indicated that this amino acid is involved in catalysis [4]. Replacement with arginine resulted in a 15-fold reduction in specific activity, with a 2-fold reduction in the K_m for PAPS, while there was no significant change of the K_m for the acceptor substrate quercetin. The slight increase in affinity of K59R for PAPS was confirmed by the results of photoaffinity labeling experiments with [^{35}S]PAPS. On the other hand, replacement with alanine resulted in a 300-fold reduction in specific activity, but the mutant enzyme retained a similar affinity for PAPS as the wild-type enzyme, indicating that Lys^{59} is not involved in cosubstrate binding. The mutant proteins were also characterized by affinity chromatography on PAP-agarose and the NaCl concentrations required for their elution were compared. Although the elution profile of the K59R mutant was similar to that of the wild-type enzyme, a significant reduction in affinity for PAP-agarose was observed for K59A, suggesting that Lys^{59} may stabilize the leaving group of the reaction. In addition, the results of comparative affinity chromatography with PAPS analogs suggest that Lys^{59} interacts with the 5′-phosphate group of PAP (Marsolais and Varin, submitted).

These results clearly indicate that Lys^{59} acts as a catalyst in the flavonol 3-ST. The slight reduction in the K_m for PAPS observed for K59R led to the proposal that in the enzyme-PAPS complex, the Lys^{59} side chain may be too distant to bind the cosubstrate, but that a weak interaction may occur with a longer arginine side chain. In the bacterial STs encoded by *nodH* from *Rhizobium meliloti* and the ORF4 of the *avrD* locus from *Pseudomonas syringae* pv. *tomato*, the Lys^{59} residue is replaced by an arginine [4] which is consistent with the partial complementation of catalytic activity observed for the arginine mutant.

The conserved region IV has the consensus sequence R(K,N)(G,A)XX(G,N)(D,G)W(K,R)XXF (Fig. 1). Hashimoto et al. first noted sequence homology between region IV and the consensus sequence GXXXXGK of the P-loop motif, known to be involved in nucleotide binding in a number of enzymes [5]. However, a distinctive feature of region IV is the presence of additional conserved residues on both sides of the segment corresponding to the P-loop motif. It was shown previously that the replacement of the two glycines and the lysine homologous to the P-loop motif with alanines in the guinea pig estrogen ST resulted in an enzyme devoid of catalytic activity that could no longer bind PAPS [6]. However, this study did not allow to evaluate the contribution of single amino acid residues. It was later shown that the replacement of any of these residues with alanine had no effect on the affinity for the cosubstrate [7]. These results are consistent with the finding that replacement of Lys^{277}, Gly^{281} or Lys^{284} with alanine in the flavonol 3-ST had a minor effect on the K_m for PAPS and catalytic activity [4].

On the other hand, the replacement of the strictly conserved residue Arg^{276} with lysine, alanine or glutamate resulted in strong reductions in PAPS affinity, as well as drastic decreases in specific activity. These results strongly suggest that Arg^{276} is involved in the formation of the enzyme-PAPS complex and indicate that this interaction is specific for the arginine side chain. In addition, the reductions in affinity for PAP-agarose observed for R276A and R276E indicate that Arg^{276} participates also in product binding, suggesting that it may be involved in catalysis.

Recently, another amino acid residue was identified that is involved in cosubstrate binding (Marsolais and Varin [8], submitted). This residue, Arg^{140} of the flavonol 3-ST, is located in the region II consensus sequence RNX(K,R)DXXVSX(Y,W)X(F,L) (Fig. 1). Conservative and non-conservative replacements at this position resulted in the loss of catalytic activity, absence of photoaffinity labeling with [^{35}S]-PAPS, and modification of the chromatographic behavior on PAP-agarose.

Amino acid sequence homology observed between cytosolic STs suggests that they evolved from a common ancestor and that their 3-D structures are similar. Since Lys^{59}, Arg^{140} and Arg^{276} are conserved in all cloned eukaryotic cytosolic STs, the results obtained with the flavonol 3-ST will most likely apply to all of these enzymes. Despite the progress in the identification of amino acid residues involved in PAPS binding and catalysis, we are still far from understanding the mechanism of sulfate transfer by these enzymes. For example, characterization of the intermediates formed during the reaction should help to interpret the results of structure-function studies.

3. Acceptor substrate binding

The flavonol 3- and 4′-STs catalyze the transfer of the sulfuryl group of PAPS to position 3 of flavonol aglycones and 4′ of flavonol 3-sulfates. To elucidate the structural aspects underlying the difference in substrate specificity observed between

these enzymes, a series of hybrid proteins were constructed by in vitro manipulation of their cloned cDNAs. Analysis of the chimeric enzyme specificities indicated that a segment of the flavonol STs, designated domain II, spanning amino acids 92–194 of the flavonol 3-ST sequence, contains all the determinants of substrate and position preferences [9] (Fig. 1). Within this domain, two subdomains of high amino acid divergence were identified by amino acid sequence comparison of STs (positions 98–110 and 153–170 of the flavonol 3-ST sequence) [9]. It is interesting to note that these subdomains are in close proximity to region II, which is involved in PAPS binding. Due to the structural similarity between all cytosolic STs, it was proposed that these variable regions could play a role in the determination of substrate specificity in all of these enzymes. This hypothesis is further supported by the amino acid sequence comparison of the human phenol-preferring and catecholamine-preferring phenol STs. Although these enzymes are 93% identical in amino acid sequence, they display distinct substrate preferences, and the variable amino acid residues are clustered within regions corresponding closely to the two subdomains of high divergence. Other protein regions might also be involved in substrate binding as suggested by a recent study on the impact of multiple mutations in the region IV of the guinea pig estrogen ST [7]. Further studies are required to define more accurately the function of the different regions in acceptor substrate recognition.

Acknowledgements

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Identification of Amino Acid Residues Critical for Catalysis and Cosubstrate Binding in the Flavonol 3-Sulfotransferase*

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The comparison of the deduced amino acid sequences of plant and animal sulfotransferases (ST) has allowed the identification of four well conserved regions, and previous experimental evidence suggested that regions I and IV might be involved in the binding of the cosubstrate, 3′-phosphoadenosine 5′-phosphosulfate (PAPS). Moreover, region IV is homologous to the glycine-rich phosphate binding loop (P-loop) motif known to be involved in nucleotide phosphate binding in several protein families. In this study, the function of amino acid residues within these two regions was investigated by site-directed mutagenesis of the plant flavonol 3-ST. In region I, our results identify Lys^{59} as critical for catalysis, since replacement of this residue with alanine resulted in a 300-fold decrease in specific activity, while a 15-fold reduction was observed after the conservative replacement with arginine. Photoaffinity labeling of K59R and K59A with [^{35}S]PAPS revealed that Lys^{59} is not required for cosubstrate binding. However, the K59A mutant had a reduced affinity for 3′-phosphoadenosine 5′-phosphate (PAP)-agarose, suggesting that Lys^{59} may participate in the stabilization of an intermediate during the reaction. In region IV, all substitutions of Arg^{276} resulted in a marked decrease in specific activity. Conservative and unconservative replacements of Arg^{276} resulted in weak photoaffinity labeling with [^{35}S]PAPS and the R276A/T73A and R276E enzymes displayed reduced affinities for PAP-agarose, suggesting that the Arg^{276} side chain is required to bind the cosubstrate. The analysis of the kinetic constants of mutant enzymes at residues Lys^{277}, Gly^{281}, and Lys^{284} allowed to confirm that region IV is involved in cosubstrate binding.

Sulfotransferases catalyze the transfer of a sulfonate group from an activated nucleotide donor, 3′-phosphoadenosine 5′-phosphosulfate (PAPS),[1] to the appropriate alcoholic or phenolic hydroxyl groups of acceptor substrates. In contrast with plant tissues in which STs have yet to be assigned a particular function, in mammals these enzymes play an important role in the detoxification of xenobiotics and endogenous metabolites, as the presence of a sulfate group increases water solubility of hydrophobic molecules and facilitates their excretion. In addition, STs are involved in the metabolic pathways of biologically active molecules, such as steroid hormones and neurotransmitters. In that case, it is generally well established that sulfate conjugation of such compounds is important to modulate their biological activity (1). Research conducted to elucidate the role of flavonoid sulfation in plants has resulted in the isolation and biochemical characterization of four position-specific STs which are involved in the stepwise formation of flavonol polysulfates (2, 3). The plant flavonol 3- and 4′-STs exhibit strict specificity for position 3 of flavonol aglycones and 4′ of flavonol 3-sulfates, and cDNA clones encoding these two enzymes were isolated and characterized (4). In a recent investigation, we constructed a series of hybrid enzymes by the substitution of protein segments between the flavonol 3- and 4′-STs. Analysis of substrate preference of the resulting chimeric proteins allowed the identification of a domain located in the central portion of these enzymes that is responsible for both substrate and position specificities (5).

Progress in understanding the structure-function relationship of STs has been limited by the fact that their three-dimensional structure has not yet been resolved. However, a large number of cDNA clones coding for STs of different specificities have been isolated from various organisms. The comparison of the deduced amino acid sequences of ST enzymes of plant and animal origin has revealed significant homology, and four well conserved regions have been identified (4, 6). These conserved regions could participate in shared functions of these enzymes, such as cosubstrate binding or specifying the proper folding for catalysis.

Two of the conserved regions of STs represent almost uninterrupted blocks of sequence identity. The conserved region I is located in the N-terminal portion of STs and its sequence is YPKSGT(T/N)W (Fig. 1). It is interesting to note that this motif is also present in two bacterial STs which, otherwise, exhibit very weak general homology with their eukaryotic functional homologs (14, 15). Recently, affinity labeling experiments with a nucleotide analog allowed the identification of two labeled amino acid residues located in the N-terminal part of the rat hepatic aryl ST IV (16). However, it is unlikely that these amino acid residues are involved in PAPS binding, since they are not conserved among all cloned STs, but their proximity to the amino acids of region I suggests that the latter may interact with the cosubstrate.

The conserved region IV, on the other hand, is located in the C-terminal portion of STs and its sequence is RK(G/A)*XX*GDWK(N/T)*X*FT. Regions sharing homology with this motif have been identified in the nonhomologous, membrane-bound *N*-heparan sulfate ST (17) and in adenosine phosphosulfate kinases (18). The motif G*XX*G*XX*K present in region IV has been

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[1] The abbreviations used are: PAPS, 3′-phosphoadenosine 5′-phosphosulfate; PAP, 3′-phosphoadenosine 5′-phosphate; ST, sulfotransferase; P-loop, the glycine-rich phosphate binding loop; bis-Tris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol; PAGE, polyacrylamide gel electrophoresis.

proposed to act as a "PAPS-binding site" because of its homology with the consensus sequence G*XXXX*GK, described as the glycine-rich phosphate binding loop (P-loop) known to be involved in nucleotide phosphate binding in a number of enzymes (19, 20). Crystal structures of adenylate kinase, $p21^{ras}$ and F_1-ATPase bound to substrate analogs have revealed that the P-loop wraps around the phosphate groups of the nucleotide and that the side chain of the invariant lysine is positioned to make contact with the β- and γ-phosphates of ATP or GTP (21–23). The critical role of the lysine residue in substrate binding has been confirmed by the results of affinity labeling and site-directed mutagenesis studies of several enzymes (20). In addition, it has been suggested that the lysine side chain is directly involved in transition state stabilization of adenylate kinase (24, 25).

The involvement of region IV in PAPS binding has recently been suggested by the results of a site-directed mutagenesis study of the guinea pig estrogen ST (26). However, since this study made use of triple mutants within the region, the contribution of discrete positions to cosubstrate binding could not be evaluated. In order to further characterize the structure and function relationship of STs, we have modified amino acid residues located in the conserved regions I and IV of the flavonol 3-ST by site-directed mutagenesis. In this paper, we describe the results of experiments which allowed to identify residues present in these two regions that are important for cosubstrate binding and catalysis.

MATERIALS AND METHODS

Site-directed Mutagenesis—Site-directed mutagenesis was performed according to the method of Kunkel (27). The pFST3 cDNA, which encodes the flavonol 3-ST, was digested with *Eco*RI and religated into the *Eco*RI site of the phage M13mp18. This construct was used to produce single-stranded template for mutagenesis experiments. Oligonucletides used for mutagenesis were for K59R: 5′-GTTATCCCAGAAGTGGCAC-3′; for K59A: 5′-GTTATCCCGCAAGTGGCAC-3′; for R276K and R276E: 5′-CTTTACTTC(A/G)(A/G)GAAGGGTAAG-3′; for R276A: 5′-CTTTACTTCGCGAAGGGTAAG-3′; for K277R and K277G: 5′-CTTCAGG(G/C)(G/C)GGGTAAG-3′; for G281A: 5′-GTAAGGATGCAGATTGGAAG-3′; for K284R and K284G: 5′-GAGATTGG(A/G)(G/C)GAACTACTTC-3′. *Escherichia coli* strain XL1-blue was transformed with the polymerization mix and single, isolated plaques were selected and screened by single strand DNA sequencing for the presence of the desired mutation. The single-stranded DNA of positive clones was amplified by the polymerase chain reaction with Vent DNA polymerase (New England Biolabs, Beverly, MA) using M13–20 and M13-reverse oligonucleotide primers. The amplified product was digested with *Eco*RI and religated into the *Eco*RI site of the plasmid pBluescript SK (Stratagene, CA). After a preliminary kinetic characterization of the mutant enzymes, the cDNAs were subcloned from pBluescript into the bacterial expression vector pQE30 (Qiagen, Chatsworth, CA) in order to facilitate the purification and kinetic analysis of the different enzymes. To this end, a *Bgl*II restriction site was introduced immediately before the first ATG codon of the cDNAs by using the oligonucleotide: 5′-GAAGATCTATGGAAGATATTATCAAAACAC-3′ (initiation codon of pFST3 is underlined). This oligonucleotide was used in conjunction with the M13–20 oligonucleotide to amplify by the polymerase chain reaction the full-length cDNAs with Vent DNA polymerase. The amplified product was digested with *Bgl*II and *Sal*I and religated into the *Bam*HI and *Sal*I sites of the pQE30 polylinker. The full-length coding sequence was determined for all mutants with strongly reduced specific activity relative to the wild-type enzyme. For all other mutants, a segment of approximately 300 base pairs containing the desired mutation was sequenced. All enzymes used for cloning were from New England Biolabs and were used under the conditions recommended.

Expression of Recombinant Sulfotransferases—An aliquot of 250 μl of an overnight culture of the *E. coli* strain XL1-blue harboring pFST3 or the different mutant constructs was used to inoculate 10 ml of LB medium. The cells were grown at 30 °C for 3 h, before the addition of the inducer, isopropyl-β-D-thiogalactopyranoside at a final concentration of 1 mM, and incubation was continued for an additional 3 h. Cells were pelleted by centrifugation and resuspended in 1 ml of 50 mM sodium phosphate, pH 8.0, 0.3 M NaCl, and 14 mM β-mercaptoethanol and lysed by sonication. Cell debris was removed by centrifugation at 12,000 × *g* for 15 min at 4 °C, and the supernatant was applied to 50 μl of nickel-nitrilotriacetic acid resin (Qiagen) preequilibrated in the same buffer. The resin was washed three times with 1 ml of 50 mM sodium phosphate, pH 6.0, 0.3 M NaCl containing 14 mM β-mercaptoethanol, and the proteins were eluted with 250 μl of the same buffer containing 150 mM imidazole. Proteins were measured by the method of Bradford (28), and bovine serum albumin was used as the standard protein.

Sulfotransferase Assay—ST activity in the protein eluates was determined immediately after purification by monitoring the incorporation of label from [^{35}S]PAPS (DuPont NEN) to the flavonol acceptor quercetin (3,5,7,3′,4′-pentahydroxyflavone) (Sigma) according to a previously described assay (29). Kinetic analysis was performed as in Ref. 30, with the following modification: the enzyme assays were performed at 25 °C in 50 mM sodium phosphate, pH 7.5. K_m values for PAPS were determined at a fixed concentration of 0.2 μM quercetin and PAPS concentrations of 1.0, 0.50, 0.25, 0.10, 0.05, and 0.01 μM. K_m values for quercetin were determined at a fixed concentration of 1.0 μM PAPS and quercetin concentrations of 0.4, 0.2, 0.1, 0.05, 0.025, and 0.016 μM. K_m and k_{cat} values were calculated from double-reciprocal Lineweaver-Burk plots using linear regression analysis. A molecular weight of 36,442 was used for the determination of k_{cat}. Kinetic constants were determined for the mutant enzymes which displayed a sufficient level of catalytic activity. As the flavonol 3-ST is subject to substrate inhibition by quercetin at concentrations above K_m, specific activities were measured at fixed concentrations of 1.0 μM PAPS and 0.2 μM quercetin.

SDS-Polyacrylamide Gel Electrophoresis—In order to verify the solubility and evaluate the level of purity of the recombinant proteins after chromatography on nickel-agarose, aliquots of the purified wild-type and mutant recombinant STs were subjected to 12% polyacrylamide gel electrophoresis according to the method of Laemmli (31). The proteins were visualized by Coomassie Blue staining.

Affinity Chromatography on PAP-Agarose—Immediately after purification on nickel-agarose, the enzyme preparations were desalted on a PD-10 column (Pharmacia, Uppsala, Sweden) preequilibrated with buffer A (25 mM bis-Tris, pH 6.8, 14 mM β-mercaptoethanol). The eluted proteins were chromatographed on a PAP-agarose column (approximately 2 ml) (Sigma) preequilibrated with buffer A and washed with 3 column volumes of the same buffer. The bound proteins were eluted with a linear gradient of 0.0 to 1 M NaCl in buffer A, at a flow rate of 0.5 ml/min, and fractions of 0.5 ml each were collected. Protein absorbance was monitored at 280 nm with a Waters 486 tunable absorbance detector. In order to obtain reproducible results, chromatography on PAP-agarose was performed with a Waters 625 LC HPLC system and a Waters ΔP minicolumn.

Immunodetection—Aliquots of the PAP-agarose affinity-purified fractions were applied onto a nitrocellulose membrane, and the dot blots were developed with polyclonal antibodies raised against the flavonol 3-ST, as described previously (30). Immunodetection was performed with an alkaline phosphatase-conjugated anti-rabbit antibody as secondary antibody and nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate as substrates.

Photoaffinity Labeling with [^{35}S]PAPS—Photoaffinity labeling with [^{35}S]PAPS was performed according to the method of Otterness *et al.* (32) with minor modifications. The reaction mixture (50 μl) contained 50 pmol of [^{35}S]PAPS and approximately 20 μg of *E. coli*-soluble protein extracted in 50 mM sodium phosphate, pH 7.5. In control experiments, 5 nmol of PAP was added as a competitor for the covalent binding of [^{35}S]PAPS to proteins. The samples were irradiated for 10 min at 4 °C in quartz microcuvettes held at a distance of 1 cm from the top plate of a UV transilluminator (model T5–36, Ultra-Violet Products, San Gabriel, CA). Aliquots of the reaction mixture were diluted with SDS sample buffer, boiled for 5 min, and submitted to SDS-PAGE electrophoresis. After electrophoresis, the proteins were electrotransferred onto a nitrocellulose membrane according to the Bio-Rad semi-dry transfer apparatus protocol and autoradiographed. To measure the migration of the recombinant STs, replicas of the gels were stained with Coomassie Blue.

DNA Sequencing and Sequence Analysis—Nucleotide sequences were determined by the dideoxy chain-termination method (33). Sequence comparison, alignment, and secondary structure prediction were performed using the Genetics Computer Group software package (34).

RESULTS

In order to identify amino acids involved in cosubstrate binding and catalysis, we modified the amino acid Lys^{59} within the

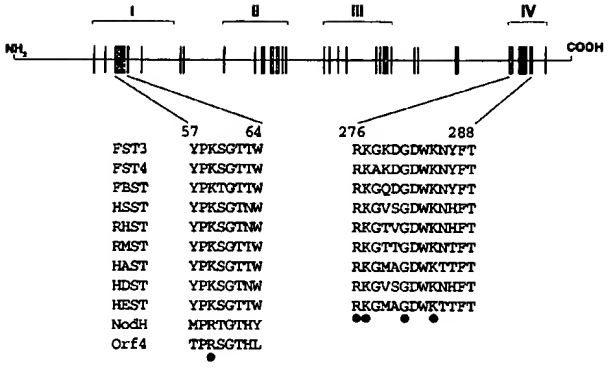

FIG. 1. **Schematic representation of the conserved regions among STs and amino acid sequence alignments of the conserved regions I and IV.** The backbone corresponds to a protein of 311 amino acids (pFST3). The amino acid sequence alignment includes the flavonol 3- and 4'-STs (*FST3* and *FST4*) with *Flaveria bidentis* ST-like cDNA (7), human liver hydroxysteroid ST (*HSST*) (8), rat hydroxysteroid ST (*RHST*) (9), rat minoxidil ST (*RMST*) (10), human aryl ST (*HAST*) (11), human dehydroepiandrosterone ST (*HDST*) (12), and human estrogen ST (*EST*) (13). Also aligned for the conserved region I are the bacterial STs amino acid sequences encoded by *nodH* from *Rhizobium meliloti* (14) and the ORF4 of the *avrD* locus from *Pseudomonas syringae* pv. tomato (15). The *boxes* indicate residues common to the nine eukaryotic STs. Position numbers refer to the flavonol 3-ST sequence. Dots indicate positions of the amino acid residues that were modified in this study. The alignment was obtained using LINEUP in the GCG package, except for NodH and ORF4, which were aligned visually.

conserved region I, and Arg^{276}, Lys^{277}, Gly^{281}, and Lys^{284} within the conserved region IV by site-directed mutagenesis of the flavonol 3-ST (Fig. 1). Conservative replacement of basic amino acids was sought, as well as nonconservative replacement with either alanine, in order to minimize structural alteration and to prevent the formation of hydrogen bonds (35), or with glycine, which eliminates side chain interactions. The recombinant wild-type and mutant enzymes were expressed in *E. coli* and were readily purified from bacterial supernatants by affinity chromatography on nickel-agarose. The recombinant proteins migrated at a distance corresponding to the predicted molecular mass of 35 kDa and were at least 95% pure, as evaluated from SDS-PAGE (data not shown). The levels of expression and stability in solution of the different proteins were comparable, suggesting that no drastic modifications of the tertiary structure were induced by the mutations. Although the recombinant flavonol 3-ST has 12 additional amino acids at its N-terminal, the kinetic properties of the nickel-agarose-purified enzyme were similar to those of the enzyme purified from the plant (Table I) (30). However, the K_m for quercetin was slightly higher (0.45 *versus* 0.20 μM for the plant enzyme) whereas the K_m for PAPS was similar (0.22 μM *versus* 0.18 μM). The k_{cat} value of 1.43 s^{-1} was representative of the value of 1.86 s^{-1} obtained with the purified plant enzyme (Table I).

In region I, replacement of Lys^{59} with alanine resulted in an approximately 300-fold reduction in specific activity. Replacement of Lys^{59} with arginine, on the other hand, resulted in an approximately 15-fold reduction in specific activity and a 2-fold decrease of the K_m for PAPS, while the K_m for quercetin was unchanged (Table I). In region IV, all mutations of Arg^{276} resulted in a pronounced decrease of catalytic activity. The cDNA encoding the mutant R276A was found to contain an additional mutation changing the unconserved Thr^{73} residue to alanine. However, since the results obtained with the double mutant R276A/T73A were consistent with those obtained for other substitutions of Arg^{276}, they were included in this study. Mutants R276K and R276A/T73A displayed approximately 5000- and 500-fold lower specific activities as compared with that of the recombinant wild-type enzyme, while mutant R276E had no detectable activity (Table I). Conservative replacement with arginine of Lys^{277} and Lys^{284}, the latter corresponding to the invariant lysine in the P-loop motif, had no significant effect on the k_{cat} or the K_m for both substrates. Nonconservative replacement of Lys^{277} and Lys^{284} with glycine gave rise to mutant proteins having a similar decrease in kcat (approximately 2–4-fold) and increase of the K_m for PAPS (approximately 6–9-fold) (Table I). The K_m for quercetin for both mutants was similar to that of the wild-type recombinant enzyme. The effect on the kinetic constants of replacing Gly^{281} with alanine was comparable with that observed for mutants K277G and K284G (Table I).

The binding properties of the inactive or very weakly active mutants toward the sulfate donor were characterized by photoaffinity labeling with [^{35}S]PAPS. UV irradiation of a crude soluble protein extract of *E. coli* harboring pFST3 in the presence of 1 μM [^{35}S]PAPS resulted in the labeling of a protein migrating at a position corresponding to that of the recombinant wild-type flavonol 3-ST (Fig. 2, *A* and *B*). As expected, the addition of a 100-fold molar excess of unlabeled PAP completely prevented labeling of the recombinant wild-type enzyme. Photoaffinity labeling of the K59A mutant was similar to that of the K59R and recombinant wild type enzymes (Fig. 2, *A* and *B*), indicating that PAPS binding is not impaired in this mutant. These results strongly suggest that although conservative and unconservative replacements of Lys^{59} have an impact on catalytic activity, this residue is not required for cosubstrate binding. In contrast, photoaffinity labeling of R276K, R276E, and R276A/T73A resulted in similar bands of very weak intensity, supporting the role of Arg^{276} in PAPS binding (Fig. 2, *A* and *B*). The intensities of the photoaffinity labeled products of K277G, G281A, and K284G were intermediate between those of the Arg^{276} mutants and recombinant wild-type enzyme (Fig. 2, *C* and *D*). These results are consistent with the 5–9-fold increases of the K_m for PAPS observed for these three mutants.

To further characterize mutants of Lys^{59} and Arg^{276} that did not have a sufficient level of catalytic activity for reliable kinetic analysis, they were submitted to affinity chromatography on PAP-agarose. The plant flavonol 3-ST is sensitive to product inhibition by PAP, a competitive inhibitor of PAPS for the active site of the enzyme, with a K_i (0.1 μM) slightly lower than the K_m for PAPS (0.18 μM) (30). As expected, the recombinant wild-type flavonol 3-ST bound strongly to the PAP-agarose affinity matrix and was eluted with 0.78 M NaCl with good reproducibility between individual experiments. The activity profile of the recombinant wild-type enzyme coincided with the elution profiles determined by monitoring the absorbance at 280 nm (Fig. 3) and by immunodetection of the purified fractions (Fig. 4). The strong interaction of the recombinant wild-type flavonol 3-ST with the PAP affinity matrix is similar to that previously observed with the plant enzyme (30).

The mutant proteins retained the ability to bind to PAP-agarose, although significant differences were observed in the salt concentration required for their elution. Mutant K59A eluted at 0.56 M NaCl, indicating a weaker affinity for PAP than the recombinant wild-type flavonol 3-ST (Figs. 3 and 4). The elution profile of the K59R mutant was similar to the recombinant wild-type enzyme, reflecting the fact that it displays only a minor change in the K_m for PAPS. Mutant R276K also eluted at the same salt concentration as the recombinant wild-type enzyme (Figs. 4 and 5). In contrast, mutants R276E and R276A/T73A eluted at a lower salt concentration of 0.64 and 0.66 M, respectively. The reduction in affinity for PAP-

TABLE I
Enzymatic properties of mutant STs

Enzyme form	K_m quercetin[a]	K_m PAPS[b]	k_{cat}[c]	k_{cat}/K_m (PAPS) $\times 10^6$	Specific activity	Relative activity
	μM		s^{-1}	s^{-1} M^{-1}	*picokatal/mg*	%
F3ST	0.20	0.18	1.86	10.3		
rF3ST	0.45	0.22	1.43	6.5	276	100
Region I						
K59R	0.43	0.10	0.07	0.7	18	6.5
K59A					0.8	0.3
Region IV						
R276K					0.06	0.02
R276E					0	0
R276A					0.6	0.2
K277R	0.57	0.32	1.17	3.7	199	72
K277G	0.43	1.34	0.34	0.2	81	29
G281A	0.56	1.17	0.52	0.4	97	35
K284R	0.69	0.25	1.61	6.4	239	87
K284G	0.84	1.91	0.59	0.3	113	41

[a] Maximum S.D. was 7% with $n = 3$.
[b] Maximum S.D. was 12% with $n = 3$.
[c] Maximum S.D. was 18% with $n = 3$.

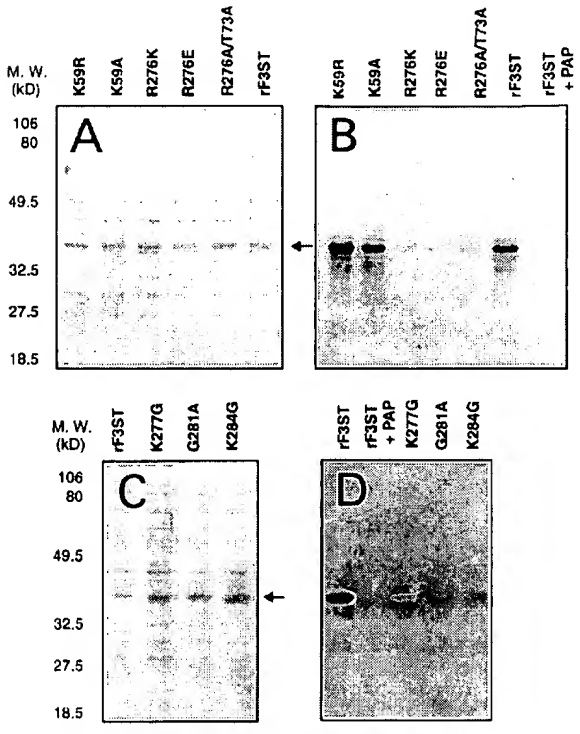

FIG. 2. **[^{35}S]PAPS photoaffinity labeling of the recombinant wild-type and mutant flavonol 3-STs.** *A*, SDS-PAGE of the protein extracts of the recombinant wild-type enzyme and of the Lys^{59} and Arg^{276} mutants after the photoaffinity labeling reaction. *B*, autoradiograph obtained with the same protein preparations shown in *A*. *C*, SDS-PAGE of the protein extracts of the recombinant wild type flavonol 3-ST and of the K277G, G281A, and K284G mutants after the photoaffinity labeling reaction. *D*, autoradiograph obtained with the same protein preparations shown in *C*. The protein band corresponding to the flavonol 3-ST is indicated by an *arrow*. *rF3ST*, histidine-tagged recombinant wild type flavonol 3-sulfotransferase.

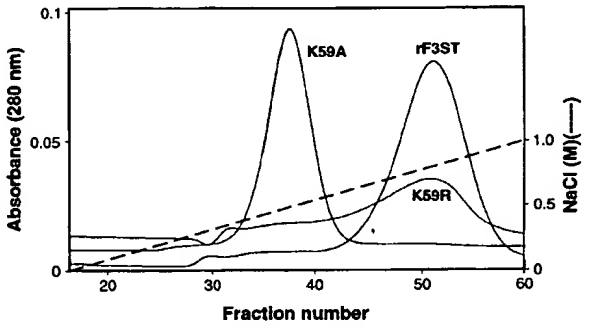

FIG. 3. **Elution profile of the recombinant wild-type, K59R, and K59A enzymes following chromatography on PAP-agarose.** *rF3ST*, histidine-tagged recombinant wild type flavonol 3-sulfotransferase.

agarose observed with mutant R276E, as compared with R276A/T73A, could be due to a charge repulsion between the phosphate groups of PAP and the carboxyl group of the glutamyl side chain. These results strongly suggest that both Lys^{59} and Arg^{276} are involved in PAP binding through ionic interactions.

Valid interpretation of the affinity chromatography data requires a prior demonstration that the affinity for PAP-agarose is specific. The following evidence suggests that the interaction with PAP-agarose is highly specific: 1) PAP-agarose affinity chromatography has already been applied to the purification of several STs, and it has been shown that they can be specifically eluted from the support by the addition of PAP or PAPS at a concentration of 1 mM or less (36–38). 2) To test whether nonspecific ionic interactions could contribute in a significant way to the affinity for PAP-agarose, control experiments were performed with mutants involving a change to the net charge of the enzyme. Mutant E101K, that displays no change in kinetic constants (data not shown), eluted at the same salt concentration as the recombinant wild-type enzyme, indicating that the introduction of a positive charge did not enhance binding to the negatively charged chromatographic support. 3) Mutants K277G and K284G, having 6–9-fold increases of the K_m for PAPS, showed only slight reductions of affinity for PAP-agarose compared to the recombinant wild-type flavonol 3-ST, eluting respectively at 0.75 and 0.77 M NaCl. Since these reductions are much smaller than those observed for K59A, R276A/T73A, and R276E, they cannot be interpreted only by the loss of a positive charge on the mutant proteins. Taken together, these results support the hypothesis that the interaction of the en-

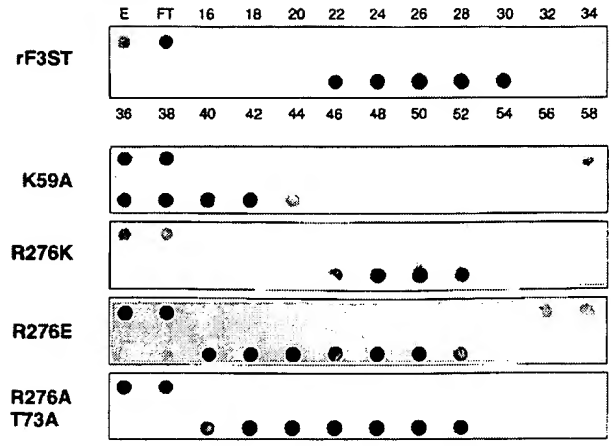

FIG. 4. **Immunoblot of the PAP-agarose purified fractions of the recombinant wild-type, K59A, R276K, R276E, and R276A/T73A enzymes with anti-*F. chloraefolia* flavonol 3-sulfotransferase (F3ST) antibodies.** The letter *E* refers to the desalted extract, and *FT* refers to the flow-through of the column. *Numbers* indicate the PAP-agarose-purified fractions. *rF3ST*, histidine-tagged recombinant wild type flavonol 3-sulfotransferase.

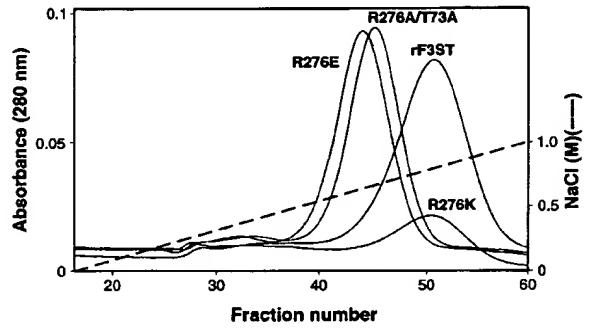

FIG. 5. **Elution profile of the recombinant wild-type, R276K, R276E, and R276A/T73A enzymes following chromatography on PAP-agarose.** *rF3ST*, histidine-tagged recombinant wild type flavonol 3-sulfotransferase.

zyme with PAP-agarose is specific. However, the technique does not allow the accurate detection of small differences in affinity for the immobilized ligand.

DISCUSSION

In this study, the function of conserved residues within regions I and IV of STs was investigated (Fig. 1). Several important features justify their choice as targets for site-directed mutagenesis. First, they represent almost uninterrupted blocks of sequence identity present in all eukaryotic cytoplasmic STs. Also, a secondary structure algorithm predicts that both regions form loop structures frequently associated with the formation of active sites. Finally, region IV is homologous to the P-loop involved in nucleotide phosphate binding in several enzymes (17).

The involvement of region I in cosubstrate binding is suggested by the recent affinity labeling of the amino acids Lys^{65} and Cys^{66} of the rat hepatic aryl ST IV by the nucleotide analog ATP dialdehyde (16). However, these amino acids are only conserved among the members of the phenol and estrogen ST families, and site-directed mutagenesis of the corresponding cysteine residue to serine in the human liver phenol ST has revealed that it is not involved in substrate binding or catalysis, but is important for the thermal stability of the enzyme (39). In view of the proximity of the affinity labeled amino acids to region I, Zheng *et al.* (16) proposed that the latter might be involved in the interaction with the cosubstrate. Our results identify Lys^{59} within this region as critical for catalysis, since replacement of this amino acid with alanine produces a pronounced decrease in specific activity. The results of photoaffinity labeling studies clearly indicate that Lys^{59} is not required for PAPS binding, since K59A is labeled to a similar extent as the K59R and recombinant wild-type enzymes, suggesting that this residue acts as a catalyst in the flavonol 3-ST. In the enzyme-PAPS complex, the Lys^{59} side chain may be too distant to interact with the cosubstrate, but when a longer arginine side chain is introduced at this position, it may interact weakly with the sulfate donor. This is consistent with the results of the affinity labeling experiments, and the small but significant reduction of the K_m for PAPS of the K59R mutant, that was reproduced in several independent experiments. On the other hand, the reduced affinity of K59A for PAP-agarose compared with that of the K59R and recombinant wild-type enzymes suggests that Lys^{59} binds a phosphate group of PAP through an ionic interaction, indicating that this residue may stabilize the leaving group of the reaction. In the absence of a proposed catalytic mechanism for STs, we can only speculate that the role of Lys^{59} may be to stabilize an intermediate and/or to lower the activation energy of a transition state.

The region IV of STs shares sequence homology with the phosphate binding loop involved in nucleotide phosphate binding in several protein families. However, a distinctive feature of region IV is the presence of additional conserved residues on both sides of the segment homologous to the P-loop motif. The function of residues specific to the ST motif (Arg^{276} and Lys^{277}) and of residues homologous to those of the P-loop motif (Gly^{281} and Lys^{284}) was investigated. All substitutions of Arg^{276} resulted in a dramatic decrease in specific activity, and the results of photoaffinity labeling studies suggest that this residue is involved in the formation of the enzyme-PAPS complex. Furthermore, the interaction is specific for the arginine side chain as demonstrated by the drastic reduction in catalytic activity of the R276K mutant. In addition, Arg^{276} is also involved in the binding of the product of the reaction as suggested by the reduction in affinity for PAP-agarose of mutants R276E and R276A/T73A. The participation of Arg^{276} in product binding suggests that it may also be involved in catalysis. These results are in agreement with the previous finding by chemical modification with phenylglyoxal that one arginine residue is required for catalysis in the rat liver phenol ST (40). We cannot exclude the possibility that structural alterations are induced by the substitutions at Lys^{59} and Arg^{276}, but the normal affinities for PAPS of the Lys^{59} mutants and the normal affinity for PAP-agarose observed for R276K suggest that there is no major change of the tertiary structure in these mutants.

The results of the kinetic analysis and photoaffinity labeling of mutants K277G, G281A, and K284G support the involvement of region IV in cosubstrate binding. Although mutations at these positions have a moderate impact on the formation of the enzyme-PAPS complex and on the catalytic activity of the enzyme, they may participate with Arg^{276} in the binding of the cosubstrate. In a recent mutational study of the guinea pig estrogen ST, it was found that the replacement with alanine of the amino acids corresponding to Gly^{278}, Gly^{281}, and Lys^{284} of the flavonol 3-ST resulted in a triple mutant with no catalytic activity that could not be photoaffinity labeled with [^{35}S]PAPS (26). Our results on Gly^{281} and Lys^{284} suggest that the absence of catalytic activity in this mutant is due to the cumulative effects of the three substitutions on cosubstrate binding. Alternatively, these residues may play a role in maintaining the proper conformation of the loop region. The effects on the

catalytic constants observed with mutants K277G, G281A, and K284G may result from subtle structural changes affecting the position of the Arg^{276} side chain.

This study represents an important step toward an understanding of catalysis in STs. Our results confirm that the conserved region IV of STs and the P-loop motif are functionally related in that both are involved in the binding of nucleotide cosubstrates. In view of the absolute conservation of the amino acids Lys^{59} and Arg^{276} in all cloned eukaryotic cytoplasmic STs, it is likely that the results presented here can be extended to all members of this class of enzymes. Other electrophilic loci may be needed in addition to Arg^{276} to stabilize the negatively charged groups of the cosubstrate, especially since PAPS is not bound by STs as a chelate complex with a divalent cation, and a residue acting as a base catalyst may be required to abstract a proton from the hydroxyl group of the acceptor substrate to activate it for nucleophilic attack at the sulfuryl group. To address these aspects of catalysis by the sulfotransferases, the construction and analysis of site-directed mutants at other conserved residues of the flavonol 3-ST are presently under progress.

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